REMARKS

A. The Status of the Claims and Specification

Claims 1-6, 8, 10, 11, 13, and 14 have been previously canceled without prejudice. Claims 7, 9, 12, 15-31, and 35 are presently canceled without prejudice. By the present amendment, claims 32-34 and 36 have been amended to claim the invention with greater particularity and specificity. The specification has been amended to eliminate minor informalities, as required by the Examiner.

No new matter has been introduced in the amendments either to the claims or to the specification. The matter introduced with the amendments was disclosed in the specification, as originally filed, and in the original claims. More specifically, claim 32 has been amended and now recites "removing glyoxal and/or methylglyoxa from one or more body fluids selected from the group consisting of blood, blood plasma and peritoneal dialysate." Support for this limitation can be found in Examples 3 and 4. In addition, instead of "the carrier of claim 23," claim 32 now recites "a carrier on which one or more biguanide agents have been immobilized". This limitation is supported by the matter disclose on page 4, line 7 of the specification. Furthermore, the biganide agents are now limited to phenformin, buformin, or pharmacologically acceptable salts thereof. Support can be found in Examples 3 and 4.

With respect to new claims 39-46, support can be found is the original claims 25, 27, 26, 28, 7, 29, 30, and 9, respectively. With respect to each of new claims 47 and 48, support for both can be found is the original claim 15.

Accordingly, entry of the amendment is respectfully requested. Upon entry of this amendment, claims 32-34 and 36-48 will be pending.

B. Priority

The Applicant acknowledges the fact that the Examiner has agreed that the effective filing date of the present application is October 6, 1999 (page 7, line 11 of the Office Action).

C. Objections to the Specification

The Examiner has objected to the specification due to minor informalities (page 2, lines 6-7 of the Office Action). The specification has been amended accordingly and now provides a correct term "butyl." It is, therefore, respectfully submitted that the objections to the specification no longer apply. Withdrawal of the objections and reconsideration are respectfully requested.

D. Objections to Claims

The Examiner has objected to claims 15, 17, 18, 29, 33, 34, and 36 due to minor informalities (page 2, lines 12-13 of the Office Action). Claims 15, 17, 18, and 29, have been canceled, and the objection is therefore moot. Claims 33, 34, and 36 have been amended and it is submitted that the objections no longer apply. Withdrawal of the objections and reconsideration are respectfully requested.

E. Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 7, 16-19, 22, 29, and 32-38 stand rejected under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite (page 3, lines 5-7 of the Office Action). The Examiner has required to amend the form of expression used in a Markush group. In addition, claim 15 stands rejected for insufficient antecedent basis for the limitation "the blood purification."

Claims 32-34 and 36 have been amended accordingly and the terminology desired by the Examiner has been now provided. Claims 7, 15-19, 22, and 29 have been canceled, and the rejection is moot with respect to these claims.

In view of the foregoing, it is respectfully submitted that the rejections under 35 U.S.C. § 112, second paragraph do not apply. Withdrawal of the rejections and reconsideration are respectfully requested.

F. Rejections Under 35 U.S.C. § 102(e)

Claims 7, 9, 23, 29, 30, and 34 stand rejected under 35 U.S.C. § 102(e) as allegedly being unpatentable over U.S. Patent No. 5,928,916 to Keogh (page 3, lines 20-21 of the Office Action). In addition, claims 37 and 38 stand rejected under 35 U.S.C. § 102(e) as allegedly being unpatentable over U.S. Patent No. 6,559,188 to Gatlin et al. (page 6, lines 7-8 of the Office Action). These rejections are respectfully traversed.

It is very well established that in order to serve as prior art, a single reference must either expressly or inherently describe each an every element as set forth in the claim sought to be patented. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ 2d. 1051, 1053 (Fed. Cir. 1987). It is submitted that Keogh fails to satisfy this requirement with respect to claims 7, 9, 23, 29, 30 and 34, and Gatlin et al. fail to satisfy this requirement with respect to claims 37 and 38.

More specifically, claims 7, 9, 23, 29 and 30 have been canceled and the rejection is moot with respect to these claims. Claim 34, as amended, now recites "a method of removing glyoxal and/or methylglyoxal from one or more body fluids." Keogh merely teaches a "biomaterial" having a charged guanidino moiety, but fails to teach, implicitly or explicitly, a method of removing glyoxal and/or methylglyoxal from one or more body fluids. Thus, Keogh does not anticipate claim 34.

With respect to the rejection of claims 37 and 38 over Gatlin et al., as an initial observation, this rejection is improper because the Examiner has not rejected the generic claim 34 over the Gatlin reference. In addition, Gatlin et al. fail to teach the use of "phenformin" or "buformin" for removing glyoxal and/or methylglyoxal from body fluids, as required by claims 37 and 38, but merely disclose an oral formulation of nateglinide or repaglinide and at least one other antidiabetic compound including metformin (see, abstract). Therefore, Gatlin et al. do not teach all the elements of claims 37 and 38.

Furthermore, Gatlin et al. do not disclose "a method of removing glyoxal and/or methylglyoxal from one or more body fluids." Gatlin et al. merely teach the use of the oral formulation of nateglinide or repaglinide and at least one other antidiabetic compound including metformin in the prevention, delay of progression or treatment of diseases (see, abstract). Even if a claimed method may comprise some steps identical to those disclosed in the prior art, an accidental or an unrecognized achievement of the method should be regarded as an element that is not anticipated by the prior art (*In re Marshall*, 578 F.2d 301, 198 USPQ 344 (CCPA 1978)). Accordingly, Gatlin et al. do not teach all the elements of claims 37 and 38.

In view the foregoing, it is submitted that the 35 U.S.C. § 102(e) rejections do not apply. Reconsideration and withdrawal of the rejections are respectfully requested.

G. Rejections Under 35 U.S.C. § 103(a)

Claims 7, 9, 12, 15-17, 19-24, and 31-37 stand rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Miyata et al. (*Kidney International*, vol. 55, pp. 389-399 (1999)), in view of Ruggiero-Lopez, et al. (*Diabetologia.*, vol. 40:A310 (1997))(page 7, line 21 through page 8, line 2 of the Office Action). In addition, claims 18, and 25-28 stand rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Miyata et al. in view of Ruggiero-Lopez et al. and further in view of Keogh (page 11, lines 13-17 of

the Office Action). Finally, claims 34-36, and 38 stand rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over U.S. Patent No. 5,952,356 to Ikeda et al. in view of Ruggiero-Lopez et al. (page 12, lines 13-15 of the Office Action). These rejections are respectfully traversed.

As discussed in the November 3, 2005, response to a previous Office Action, to establish a *prima facie* case of obviousness, (1) there must be some suggestion or motivation to modify the reference(s) as proposed by the Examiner; (2) there must be a reasonable expectation of success and (3) the prior art reference(s) must teach or suggest all of the claim limitations. The Applicants respectfully submit that none of the cited references, either alone or in combination, disclose or suggest every limitation of the pending claims.

Specifically, claims 7, 12, 15, and 23 have been canceled and the rejection is therefore moot with respect to these claims. Claim 34 has been amended and now recites a method of removing glyoxal and/or methylglyoxal from one or more body fluids, comprising contacting one or more biguanide agents or a carrier having the biguanide agents with the body fluids, wherein the biguanide agents are selected from the group consisting of phenformin, buformin, and pharmacologically acceptable salts thereof.

It is submitted that neither Miyataet al. nor Ruggiero-Lopez et al. teaches or suggests the use of phenformin, buformin, or pharmacologically acceptable salts thereof. The use of phenformin, buformin, or pharmacologically acceptable salts thereof is an element of the amended claims. Thus, combination of Miyata et al. and Ruggiero-Lopez et al. does not teach or suggest all of the claim limitations.

The Examiner has stated that "a skilled artisan ... would have had a reasonable expectation of success in using carriers containing immobilized biguanide agents to remove AGEs, because Ruggiero-Lopez' results suggest metformin consumes reactive carbonyl compounds that lead to the formation of AGEs and aminoguanidine is a well

known carbonyl stress inhibitor" (sentence bridging page 10 and 11). The Applicant respectfully disagrees. It should be noted that an amino-rich property of a compound does not necessarily suggest the activity of removing carbonyl compounds. For example, Figure 22 of U.S. Patent No. 6,919,326 (hereafter, "the '326 patent") shows that aminoguanidine can effectively remove 3-deoxyglucosone. However, metformin did not effectively remove 3-deoxyglucosone (see, FIG. 19 of the '326 patent). This discrepancy between the two amino-rich compounds shows that structural similarity of the compounds alone is not sufficient to suggest the activity of removing carbonyl compounds. Accordingly, there was no reasonable expectation of success that phenformin or buformin would effectively remove glyoxal and/or methylglyoxal.

In addition, it was unpredictable whether phenformin and buformin can effectively remove glyoxal and/or methylglyoxal in "body fluids." The art would have recognized that many chemical or biological reactions which take place effectively in optimal conditions, are interfered in the presence of body fluids such as serum. For example, Lehnert et al. (Eur. J. Cancer, 1996, 32A: 862-867), a copy of which is enclosed for easy reference, teach that many chemosensitizers (CS) are inactivated by serum (see Appendix 1). They specifically describe that "when the CS were used at concentrations achievable in humans, the activity of all agents except quinine was markedly reduced by serum" (see abstract). Leung et al. (Am. J. Gastroenterol., 1998, 93: 1914-1918; enclosed (see Appendix 2)) teach that a biopsy urease test (BUT) is interfered in presence of blood. They describe that "the color change of the microtiter urease test was significantly reduced by blood" (abstract). Arakaki et al. (FEMS Immunol. Med. Microbiol., 1998, 22: 283-291; enclosed (see Appendix 3)) also teach that the biological activity of lipoteichoic acid (LTA) is inhibited by serum (see title).

The above-described references suggest that serum or serum components often inhibit chemical or biological reactions. Accordingly, even if chemical or biological reactions are carried out in optimal condition using an artificial reaction buffer, it is

unpredictable whether the reactions would similarly take place in blood or blood plasma. The present invention has demonstrated that phenformin and buformin can effectively remove glyoxal and/or methylglyoxal in body fluids (Figs. 8 and 9). These results would not have been predictable from the combination of Miyata et al. and Ruggiero-Lopezet al., because these publications do not teach or suggest the ability of any biguanide to remove glyoxal and methylglyoxal in body fluids.

In view of the foregoing, it is respectfully submitted that claim 34 is patentably distinguishable over Miyata et al. in view of Ruggiero-Lopez, et al.

With respect to the rejection of claims 18 and 25-30, claims 18 and 25-30 have been canceled and the rejection is therefore moot with respect to these claims.

With respect to the rejection of claims 34-36, and 38, according to the Examiner, a combination of Ikeda et al. and Ruggiero-Lopez et al. can be used to establish a *prima facie* case of obviousness. The Applicant respectfully disagrees. Ikeda et al. teach pharmaceutical compositions comprising insulin sensitivity enhancer in combination with other antidiabetics for prophylaxis and treatment of diabetes (see, abstract). On the other hand, Ruggiero-Lopez et al. teach the use of metformin for preventing glycoxidation of albumin by dicarbonyl compounds and decreasing AGE formation. Therefore, Ikeda et al. teach the use for biguanides (improving insulin sensitivity) that is different from the use proposed by Ruggiero-Lopez et al. (inhibiting glycoxidation). Thus, it is submitted that no motivation exists for combining Ikeda et al. and Ruggiero-Lopez et al.

In addition, claims 34-36 and 38, as amended, are directed to the method of removing glyoxal and/or methylglyoxal by phenformin, buformin, and pharmacologically acceptable salts thereof. Neither Ikeda et al. nor Ruggiero-Lopez et al. teaches that phenformin or buformin have the ability to remove glyoxal and methylglyoxal. As mentioned above, structural similarity of the compounds does not necessarily mean similar activity. Therefore, the removal of glyoxal and methylglyoxal by phenformin or

buformin is an unrecognized achievement, which should be regarded as a limitation of the claims (see, *In re Marshall*, *supra*). Furthermore, no evidence is shown in these references whether phenformin or buformin can effectively remove glyoxal and methylglyoxal in body fluids. Accordingly, the combination of Ikeda et al. and Ruggiero-Lopez et al. fails to teach or suggest all of the limitations of the claims.

In view of the foregoing, it is submitted that the 35 U.S.C. § 103(a) rejections do not apply. Reconsideration and withdrawal of the rejection are respectfully requested.

H. Double Patenting Rejection

Claims 15, 23, 32, and 34 have been rejected under the non-statutory, judicially created doctrine of obviousness-type double patenting over the claims 1 and 2 of U.S. patent No. 6,919,326 in view of Ruggiero-Lopez et al. (page 16 of the Office Action). While the Applicant respectfully traverses this rejection, it is believed that this issue has become moot in view of the terminal disclaimer which accompanies this response. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

In view of the above remarks, reconsideration and favorable action on all claims are respectfully requested. In the event any matters remain to be resolved, the Examiner is requested to contact the undersigned at the telephone number given below so that a prompt disposition of this application can be achieved.

Check number 581815 in the amount of $120.00 is enclosed as payment for the one-month Petition for Extension of time fee, and check number 581835 in the amount of $130.00 is enclosed as payment for the Terminal Disclaimer. Applicants do not believe any other fees are due in connection with this Amendment in Response to the Office Action. However, the Commissioner is hereby authorized to charge any fees required by this submission, or credit any overpayments, to Deposit Account No. 07-1896 referencing the above-identified docket number. A duplicate copy this Transmittal Sheet is enclosed.

Respectfully submitted,

Date: May 12, 2006

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APPENDIX

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GA8005465

Pergamon

European Journal of Cancer Vol. 32A, No. 5, pp. 862–867, 1996

Printed in Great Britain
0959–8049/96 $15.00+0.00

S0959-8049(96)00004-4

Original Paper

Serum Can Inhibit Reversal of Multidrug Resistance by Chemosensitisers

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The purpose of this study was to evaluate to what extent the ability of various chemosensitisers (CS) to reverse P-glycoprotein-associated multidrug resistance (MDR) is reduced when tested in physiological serum protein concentrations. Utilising drug sensitivity and accumulation assays, the CS were tested in medium containing 10% fetal bovine serum and in 100% horse or human serum. Two RPMI 8226 human myeloma sublines were used which express different levels of P-glycoprotein. The CS were tested at various concentrations, including clinically achievable blood levels. When using the CS at high doses, wide differences were observed in the extent CS activity was diminished by serum. Verapamil, cyclosporin A and quinine were not affected, quinidine and medroxyprogesterone acetate were moderately inhibited, and amiodarone and trifluoperazine were largely inactivated. When the CS were used at concentrations achievable in humans, the activity of all agents except quinine was markedly reduced by serum. With respect to the extent to which CS activity was diminished by serum, good statistical correlation ($r > 0.90$, $P < 0.001$) was found between the use of cytotoxicity and drug accumulation assays, horse and human serum or cell lines with high and low levels of P-glycoprotein, respectively. These studies demonstrated that physiological serum protein concentrations can profoundly diminish the MDR reversing activity of particular CS. Some drugs, such as amiodarone and trifluoperazine, are largely inactivated by serum when used at a wide range of concentrations. Other agents, such as verapamil and cyclosporin A, are essentially unaffected when used at high doses but markedly inhibited at concentrations achievable in humans. These data suggest that *in vitro* studies of CS in medium containing low serum protein concentrations can result in misleading conclusions regarding the potential clinical activity of such agents.

Key words: multidrug resistance, chemosensitisers, serum inhibition
Eur J Cancer, Vol. 32A, No. 5, pp. 862–867, 1996

INTRODUCTION

P-GLYCOPROTEIN (Pgp)-associated multidrug resistance (MDR) is an extensively studied experimental phenomenon [1] that seems to have clinical relevance. Overexpression of MDR1/Pgp has been detected in a variety of human cancers [2, 3] and in certain malignancies, such as acute leukaemias and various childhood cancers, MDR1/Pgp-positivity has been found to correlate with a lower response rate to chemotherapy and shorter relapse-free and overall survival [4–6]. Hence, effective clinical reversal of MDR may be able to improve chemotherapy efficacy in such cancers.

A number of agents have been found capable of overcoming MDR in preclinical models [7]. Such chemosensitisers (CS) are thought to function by competitive inhibition of Pgp-mediated efflux of cytotoxic drugs. In recent years, a variety of CS have been used in clinical studies aimed at overcoming MDR, including verapamil (VER), cyclosporin A (CSA), quinidine (QD), quinine (Q), amiodarone (AMD), trifluoperazine (TFP) and tamoxifen (TAM) [8–16]. In most of these studies, CS have failed to show activity in overcoming clinical chemotherapy resistance [17, 18]. Thus, better preclinical models for assessing potential clinical activity of CS appear to be needed.

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Revised 26 Oct. 1995; accepted 20 Nov. 1995.

In vitro studies of CS are usually performed in medium containing low serum concentrations. In such conditions, a large proportion of any agent, irrespective of its degree of serum protein binding, is present as a free, non-protein-bound drug capable of blocking Pgp function. Clinically, however, CS are exposed to high concentrations of drug-binding proteins, both in blood and tissues. Depending on the degree of serum protein binding, this can significantly reduce bioavailability of CS, which may diminish MDR reversing activity. The goal of the present study was to evaluate to what extent the activity of various first-generation CS are inhibited when used in physiological serum protein concentrations. Further objectives were to assess whether the order of equimolar potency of these agents differs when determined in low versus high serum protein concentrations and to devise a suitable model for analysing serum effects on CS.

MATERIALS AND METHODS

Cells

The 8226/DOX6 and DOX40 lines, which were derived from the RPMI 8226 human myeloma cell line and exhibit all the characteristics of MDR as previously described [19], were used as MDR models in these studies. The 8226/DOX6 cells express Pgp at a level similar to myeloma cells from patients with drug-refractory disease [20]. The amount of Pgp present in 8226/DOX40 variants is higher than usually detected in clinical tumour samples. Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin, streptomycin and L-glutamine (1% v/v). Cell cultures were maintained at 37°C in humidified air supplemented with 6% CO_2. Prior to experiments, 8226/DOX cells were grown without doxorubicin (DOX) for a minimum of 1 week. Using the Hoechst dye 33256, cell lines were proven to be free of *Mycoplasma* infection prior to experiments [21].

Drugs and sera

The formulations available for clinical i.v. use were utilised as sources of DOX, daunorubicin (DNR), VER, CSA, AMD, TFP and medroxyprogesterone acetate (MPA). QD, Q and TAM were purchased in powder form from Sigma (St. Louis, Missouri, U.S.A. and Buchs, Switzerland). The source of CSA was Sandimmun® IV, which contains Cremophor® EL as vehicle for the water-insoluble CSA. Cremophor® EL has been previously found to be capable of reversing MDR *in vitro* [22].

FBS was purchased from Irvine Scientific (Santa Ana, California, U.S.A.) or GIBCO BRL (Life Technologies AG, Basel, Switzerland). Horse serum was obtained from Irvine Scientific or Inotech AG (Dottikon, Switzerland), human serum from healthy volunteers. Only those lots of horse serum which were shown by biochemical analysis to have a serum protein profile (total protein, albumin, globulin subfractions) similar to normal human serum were used. The concentration of alpha-1-acid glycoprotein (AAG) in the human sera was in the normal range. Owing to the lack of species-specific reagents, we were not able to measure AAG concentrations in horse serum.

In vitro *sensitivity testing*

CS effects on DOX sensitivity were tested by using a two-layer, soft-agar colony assay [23]. Exponentially growing cells were exposed to drug(s) for 1 h at 37°C in either RPMI 1640 medium containing 10% FBS or in 100% horse or human serum. Cells were then washed twice with ice-cold phosphate-buffered-saline (PBS) and plated in triplicate at a concentration of 10000 cells per 35 mm tissue culture dish. Tumour cell colonies of 60 μm in diameter or greater were enumerated 14–21 days after plating using an automated image analysis instrument optimised for tumour colony counting (FAS II Omnicon, Bausch and Lomb, Rochester, New York, U.S.A.) [24]. The per cent survival was determined from the plating efficiencies of treated versus control cells. The IC_{50} for DOX, alone or in combination with CS, was defined as the DOX concentration which reduced colony formation to 50% of untreated controls. Sensitisation factors (SFs) were determined by dividing the IC_{50} for DOX alone by the IC_{50} for DOX in the presence of a particular CS.

Drug accumulation studies

CS effects on cellular DNR accumulation were analysed by flow cytometry (FACScan or FACStar, Becton Dickinson, San Jose, California, U.S.A.). DNR rather than DOX was used as the index anthracycline because of its superior fluorescence profile and, thus, good correlation of intracellular drug concentrations as measured by flow cytometry and other methods, such as high-pressure liquid chromatography [25]. Preliminary experiments indicated that the fluorescence spectra of the CS did not interfere with the fluorescence emitted by DNR.

Exponentially growing cells were incubated at a concentration of 500000 cells/ml at 37°C with DNR, in the absence or presence of a CS. DNR concentrations were 1.5 and 3.0 μM when using 8226/DOX6 and DOX40 cells, respectively. After drug exposure for 1 h in either medium containing 10% FBS or 100% horse or human serum, cells were washed twice with ice-cold PBS, resuspended in ice-cold PBS, and kept on ice until analysis of cellular DNR content. The excitation and emission wavelengths used were 488 and 585 nm, respectively. Ten thousand cells were analysed for each histogram generated. In preliminary experiments, cellular uptake of DNR was found to be diminished by approximately 75% when using 100% serum rather than culture medium supplemented with 10% FBS.

Statistical methods

The cytotoxicity data were analysed assuming a Poisson distribution for cell colony growth. Maximum likelihood estimates of the regression parameters were computed using a Newton–Raphson algorithm in the statistical language S [26]. Estimates of the standard errors for the parameters were obtained using asymptotic likelihood theory [27]. Standard errors for IC_{50}s, SFs and reduction ratios were obtained by applying the delta method [28]. Means and standard errors for DNR accumulation were computer-based on logarithmic transformation of the data. Correlations between SFs for different types and concentrations of sera and between the reduction ratios for DOX cytotoxicity and DNR accumulation were calculated using Pearson's correlation coefficient. *P* values were based on the one-sample *t*-test for correlation coefficients [29]. Statistical comparison of SFs as achieved by CS in 100% serum versus culture medium was performed by using the non-parametric Wilcoxon test.

RESULTS

First, we evaluated the effects of horse serum (with protein composition similar to human serum) on the ability of CS

Table 1. Chemosensitiser-effects on doxorubicin IC_{50}(μM) in 8226/DOX40 cells in culture medium containing 10% fetal bovine serum versus in 100% horse serum

Drug	Culture medium			100% horse serum		
	Estimate*	95% confidence interval	Mean SF†	Estimate*	95% confidence interval	Mean SF†
Doxorubicin alone	3.86	3.62–4.09		15.45	14.63–16.55	
+ Verapamil‡	0.37	0.33–0.42	10.4	1.42	1.32–1.51	10.9
+ Cyclosporin A	0.32	0.29–0.35	12.0	1.30	1.16–1.42	11.9
+ Amiodarone	0.17	0.15–0.20	22.2§	4.56	4.36–4.70	3.4§
+ Quinidine	0.71	0.62–0.77	5.4§	3.95	3.77–4.11	3.9§
+ Quinine	0.88	0.78–0.97	4.4	2.97	2.82–3.11	5.2
+ Trifluoperazine	0.30	0.26–0.33	12.9§	7.72	7.43–8.01	2.0§
+ Tamoxifen	2.63	2.44–2.80	1.5	10.89	10.31–11.26	1.4
+ Medroxyprogesterone acetate	0.45	0.39–0.53	8.5§	2.39	2.07–2.67	6.5§

*Estimates and 95% confidence intervals derived from two independent experiments performed in triplicate. †SF, sensitisation factor. ‡Each chemosensitiser used at 20 μM. §$P = <0.005$.

to enhance anthracycline cytotoxicity and accumulation in 8226/DOX40 cells. Table 1 shows the effects of eight CS on DOX sensitivity when used at 20 μM in medium containing 10% FBS or in 100% horse serum. CS were non-toxic to the cells when used alone. CS effects on DNR accumulation are illustrated in Figure 1. In both assay systems, the use of 100% serum almost completely inactivated AMD and TFP, resulted in moderate inhibition of QD and MPA, and had no effect on VER, CSA and Q. Accordingly, the order of equimolar CS activity changed significantly in 100% versus 10% serum. Excellent agreement was found between flow cytometry and clonogenic assay with respect to the extent of CS inhibition produced by serum (Figure 2).

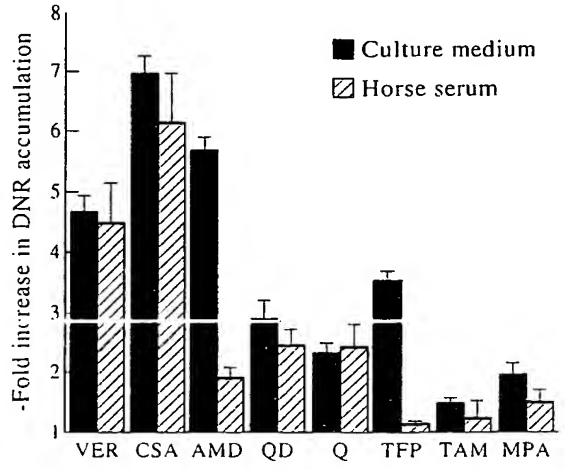

Figure 1. Effects of chemosensitisers on daunorubicin accumulation in 8226/DOX40 cells when used in culture medium containing 10% FBS or in 100% horse serum. Cells were incubated with 3 μM DNR alone or in the presence of a CS at 20 μM. Each column represents mean ± SEM of three independent experiments each performed in triplicate. VER, verapamil; CSA, cyclosporin A; AMD, amiodarone; QD, quinidine; Q, quinine; TFP, trifluoperazine; TAM, tamoxifen; MPA, medroxyprogesterone acetate.

Escalating concentrations of AAG have been recently found to render VER inactive in cells with high but not with low levels of Pgp [30]. Accordingly, the 8226/DOX6 cell line was used in subsequent experiments, which expresses low amounts of Pgp. To further enhance clinical relevance, horse serum was replaced by human serum. Table 2 and Figure 3, respectively, show the effects of 100% serum on the ability of CS to enhance DOX cytotoxicity and DNR accumulation. The pattern of CS inhibition was similar to that observed with horse serum. However, in human serum, the activity of all CS was diminished to some extent. In these experiments, CS concentrations of 5.0 rather than 20 μM, and 8226/DOX6 rather than DOX40 cells, were used. Thus, it was not clear whether the observed differences in the extent of CS inhibition were due to the use of human serum, lower CS doses or cells with lower amounts of Pgp. To answer that question,

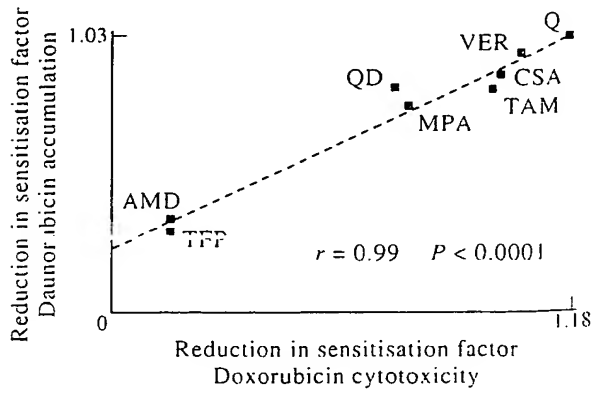

Figure 2. Correlation of reduction in chemosensitiser activity on anthracycline cytotoxicity versus accumulation, as produced by horse serum. CS were used at 20 μM. Reduction ratios were derived from mean sensitisation factors as calculated for CS in 100% serum versus culture medium. Symbols represent mean reduction ratios of at least three independent experiments each performed in triplicate. See Figure 1 legend for abbreviations.

Table 2. Chemosensitiser effects on doxorubicin IC_{50}(μM) in 8226/DOX6 cells in culture medium containing 10% fetal bovine serum versus in 100% human serum

Drug	Culture medium			100% human serum		
	Estimate*	95% confidence interval	Mean SF†	Estimate*	95% confidence interval	Mean SF†
Doxorubicin alone	0.25	0.22–0.29		0.60	0.52–0.68	
+ Verapamil‡	0.05	0.04–0.06	5.1§	0.21	0.18–0.24	2.8
+ Cyclosporin A	0.03	0.03–0.04	7.7	0.17	0.15–0.20	3.5
+ Amiodarone	0.04	0.04–0.05	6.2	0.47	0.41–0.55	1.3
+ Quinidine	0.08	0.07–0.09	3.3	0.39	0.34–0.45	1.5
+ Quinine	0.08	0.07–0.09	3.3	0.33	0.28–0.38	1.8
+ Trifluoperazine	0.06	0.05–0.07	4.2	0.58	0.50–0.66	1.0
+ Medroxyprogesterone acetate	0.06	0.05–0.07	4.1	0.50	0.45–0.56	1.2

*Estimates and 95% confidence intervals derived from two independent experiments performed in triplicate. †SF, sensitisation factor. ‡Each chemosensitiser used at 5 μM. §For each chemosensitiser tested, difference of SF in human serum versus culture medium was significant ($P = <0.005$).

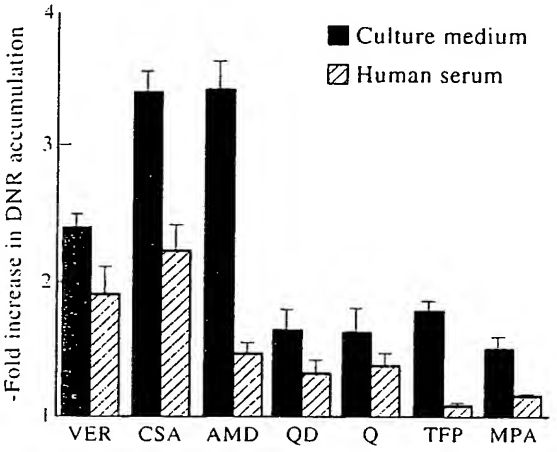

Figure 3. Effects of chemosensitisers on daunorubicin accumulation in 8226/DOX6 cells when used in culture medium containing 10% FBS versus in 100% human serum. Cells were incubated with 1.5 μM DNR alone or in the presence of CS at 5 μM. Each column represents mean ± SEM of at least two independent experiments each performed in triplicate. See Figure 1 legend for abbreviations.

clonogenic as well as flow cytometry studies were repeated by using 8226/DOX40 versus DOX6 cells, CS at 5.0 versus 20 μM, and 100% human versus horse serum. Similar levels of serum inhibition were found when using 8226/DOX40 or DOX6 cells and horse or human serum, with correlation coefficients of >0.90 and P values <0.001 (data not shown). In contrast, diminution of CS activity was greater when the agents were used at 5.0 rather than 20 μM. This particular observation appeared important because for most of these CS the serum levels achievable in humans are lower than the concentrations used in these studies. Thus, we evaluated the extent of inhibition by human serum when using VER, Q and AMD at graded concentrations, including clinically achievable blood levels (Figure 4). While VER and Q were not affected by 100% serum when used at 20 μM, an increasing and eventually profound reduction in reversing activity was observed when used at lower doses. Conversely, AMD was profoundly inhibited by serum at each dose level tested.

DISCUSSION

Among researchers working in the field of MDR reversal, there is a general feeling that better preclinical models are needed for assessing the potential clinical effectiveness of CS. This notion is based on the fact that while many agents seem capable of effectively reversing MDR in preclinical systems, they are frequently unable to overcome clinical drug resistance. Obviously, the biological basis of drug resistance is often poorly understood in clinical cancers where overexpression of MDR1/Pgp may be but one of many factors or even irrelevant. However, there are obvious differences between *in vitro* and clinical CS studies which can be addressed. Two such factors are the concentrations of CS, which in experimental studies often are much higher than achievable in humans, and the concentrations of drug-binding proteins that CS are exposed to, which *in vitro* are usually much lower than present in the body. As a result of both these factors, the amount of free CS available in clinical MDR reversal studies may be significantly lower than *in vitro*.

Our data indicate that some CS can be significantly inhibited by serum. AMD and TFP were largely inactivated even when used at doses which were highly effective in overcoming MDR when tested in medium containing low serum protein concentrations. VER, CSA and Q, in contrast, were little affected by serum when used at high doses. The differential extent of inhibition by serum significantly changed the order of MDR reversing potency when CS were used in high versus low serum concentrations. The particular differences observed in serum inhibition could not have been predicted from the degrees of serum protein binding reported for the various CS, which are around 99, 96, 98 and 90% for TFP [31], AMD [32], CSA [33] and VER [34], respectively. Furthermore, for various drugs, the published data on protein binding differ depending on the particular type of analytic method used. For example, the degrees of serum protein binding reported for QD and Q vary from 50 to 95% and from 70 to 91%, respectively [35, 36]. The concentration of free CS present at the various experimental conditions employed in these studies was not analysed. However, it seems quite likely that the loss in activity observed for particular CS in high serum concentrations was due to protein-binding of the agents.

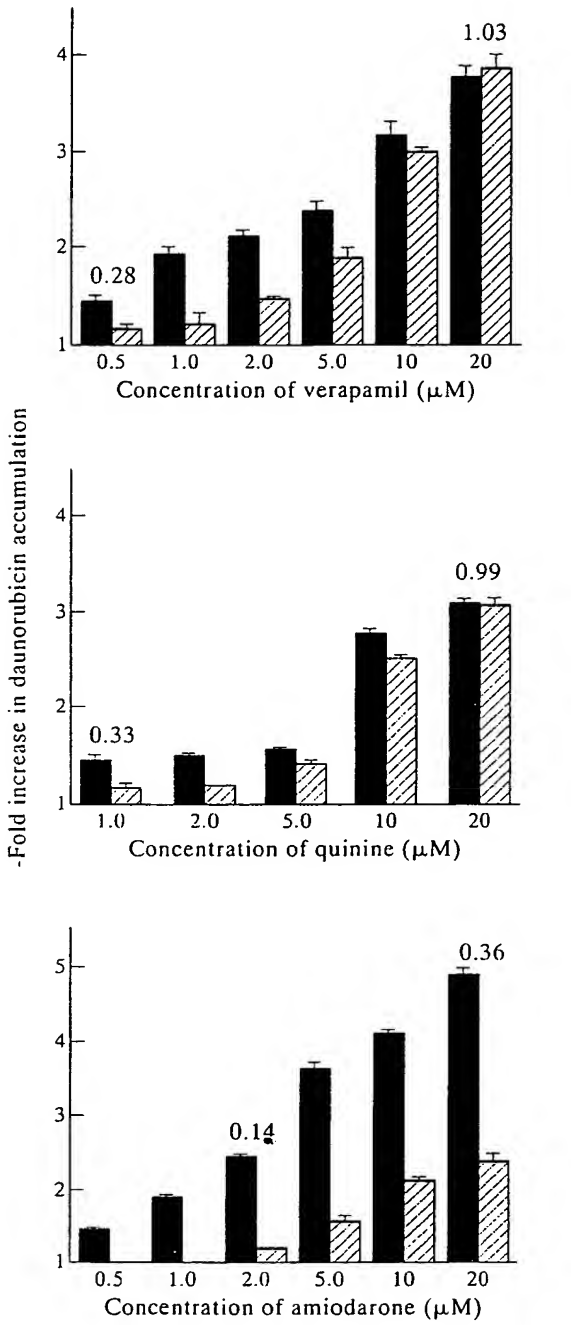

Figure 4. Effects of graded concentrations of verapamil, quinine and amiodarone on DNR accumulation in 8226/DOX6 cells. Cells were incubated with 1.5 μM DNR alone or in the presence of CS at the indicated concentrations in culture medium containing 10% FBS (solid columns) or in 100% human serum (hatched columns). The numbers shown on top of various pairs of columns represent mean reduction ratios in 100% serum versus culture medium containing 10% serum as calculated for the particular CS concentrations. Amiodarone at 0.5 and 1.0 μM was inactive when used in 100% human serum. Columns represent mean ± SEM of at least two independent experiments each performed in triplicate.

Broxterman and associates have previously found VER activity to be reduced by 40–50% when tested in 4% bovine serum albumin (BSA) or 4% human plasma versus 1% BSA [37]. A good correlation between cytotoxicity and drug accumulation assays was observed with respect to the degree of CS inhibition by protein binding, which is in agreement with our findings. The *in vitro* effects of rat serum on the ability of CS to enhance DOX uptake in a Pgp-positive rat colon cancer cell line have been reported by Genne and colleagues [38]. The study focused on *cinchona* alkaloids, including quinine and quinidine. However, amiodarone and verapamil were also tested. In agreement with our observations, amiodarone activity was profoundly diminished by rat serum. Quinine and quinidine were little affected, while verapamil activity was reduced by approximately 50%. Recently, the effects of AAG on the MDR reversal activities of VER and toremifene have been reported. Increasing AAG concentrations have been found to diminish progressively the ability of VER and toremifene to reverse resistance in a MDR cell line expressing high levels of Pgp [30, 39]. At an AAG concentration of 2 mg/ml, a level which can be present in human serum, VER activity was fully abrogated in the MDR subline, while it was reduced by only 20% in the wild-type cells which express low levels of Pgp. In the present study, no differences were found in the degree of CS inhibition by serum when using 8226/DOX40 and DOX6 cells, two MDR lines which express high and low amounts of Pgp, respectively. Most CS are hydrophobic bases at physiological pH. Such agents are bound in serum to various types of proteins including albumin, alpha-2-globulin and AAG [35]. Thus, we believe that testing of CS in whole serum is better able to approximate clinical conditions compared with using medium supplemented with particular serum proteins such as albumin or AAG.

The extent of inhibition of CS activity was similar when using horse or human serum and flow cytometry or clonogenic assay, respectively. Thus, flow cytometry and horse serum appear to be valid tools for evaluating serum effects on CS. A distinct advantage of flow cytometry over drug sensitivity assays is that results are available within a few hours. The advantage of horse serum over commercially available human serum are the much lower costs. It should be noted that wide variations in protein concentrations were found in various lots of horse serum, which resulted in differing degrees of CS inhibition compared with human serum (data not shown). Thus, analysis of protein concentrations prior to use of horse serum in such studies is critical.

When used at low concentrations, each of the tested CS was profoundly inhibited by serum. It is to be recognised that for most of the agents evaluated in the present study, the maximum achievable serum levels in humans are in the range of 2–5 μM or even lower, that is, in a range of concentrations where serum diminished MDR reversal activity of either drug. For instance, maximum achievable blood levels of racemic VER have been previously found to be around 2 μM [40]. At this particular dose level, serum diminished VER activity by approximately 50%. By contrast, Q is able to readily yield blood levels of 20 μM [41], a concentration at which Q activity was not impaired by serum. When tested in 100% human serum, Q at 20 μM was approximately twice as effective as VER at 2 μM in increasing DNR accumulation in 8226/DOX6 cells. Whether this suggests Q is more likely than VER to be capable of overcoming clinical drug resistance remains to be proven, and these *in vitro* data must be interpreted with great caution with respect to their clinical application.

We recognise that any kind of experimental model falls short of truely mimicking the complex clinical scenario. How-

ever, we believe that *in vitro* models can be developed which approximate clinical conditions more closely than the assays which are routinely applied, for example, by testing the CS at clinically relevant concentrations in an environment containing high serum concentrations. Studies along these lines are currently in progress and we hope that such pharmacologically guided *in vitro* modelling will prove capable of better predicting the potential clinical effectiveness of CS.

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Acknowledgements—This work was supported by grant No. 31-37521.93 from the Swiss National Science Foundation, Berne, Switzerland, grants CA 17094, 23074 and 43043 from the National Institutes of Health, Bethesda, Maryland, and grant No. 82-9301 from The Arizona Disease Control Commission, Phoenix, U.S.A. Manfred Lehnert was the recipient of grants J0316-MED and J0488-MED from the Erwin-Schroedinger-Foundation, Fonds zur Foerderung der Wissenschaftlichen Forschung, Vienna, Austria.

APPENDIX

2

THE AMERICAN JOURNAL OF GASTROENTEROLOGY

Published by Elsevier Science Inc.

Vol. 93, No. 10, 1998
ISSN 0002-9270/98/$19.00
PII S0002-9270(98)00338-4

False-Negative Biopsy Urease Test in Bleeding Ulcers Caused by the Buffering Effects of Blood

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Objectives: **A false-negative biopsy urease test (BUT) is common in *Helicobacter pylori*-associated bleeding peptic ulcers. Although blood in the stomach is thought to interfere with the biopsy urease test, the underlying mechanism remains unknown. This *in vitro* experiment sought to identify the blood component(s) that interfere with the biopsy urease test, and delineate the mechanism of inhibition.** *Methods:* **The modified Hazell's microtiter test was used to detect the urease activity of *H. pylori*. A positive result was indicated by a color change of the pH indicator, bromothymol blue, at 630 nm. Human whole blood, sera with and without anti-*H. pylori* antibody, electrolytes, and enzymes were incubated with *H. pylori* to identify the blood component(s) causing the inhibition of urease activity. In addition, any interference of the pH color indicator was tested by adding different concentrations of serum albumin to the urease reagent that contained a fixed quantity of ammonia in the absence of *H. pylori*.** *Results:* **The color change of the microtiter urease test was significantly reduced by blood ($p < 0.0001$), regardless of the presence of anti-*H. pylori* antibody. Electrolytes and serum enzymes did not interfere with the urease test. The color change of the pH indicator was progressively suppressed by higher concentrations of serum albumin.** *Conclusions:* **Blood adversely affects the performance of the BUT. This is mediated by the buffering effect of serum albumin on the pH indicator, rather than by a direct inhibition on the urease activity. (Am J Gastroenterol 1998;93:1914–1918. © 1998 by Am. Coll. of Gastroenterology)**

INTRODUCTION

Unlike nonbleeding ulcers, bleeding ulcers appear to have a lower prevalence of *Helicobacter pylori* infection (1). Using the biopsy urease test (BUT), we have previously shown that *H. pylori* was detected in 93% of nonbleeding duodenal ulcers but in only 71% of bleeding duodenal ulcers (1). This discrepancy cannot be fully accounted for by the use of nonsteroidal antiinflammatory drugs. Although the BUT is known to be a highly accurate test for *H. pylori*, recent studies revealed a relatively low sensitivity of the BUT in bleeding ulcers (2–6). There were cases where *H. pylori* was identified by histology but failed to be detected by the BUT. False-negative results ranging from 25% to 69% have been reported. Although blood in the stomach has been implicated as the culprit, the underlying mechanism remains unclear (5).

There are three possible mechanisms by which blood could interfere with the performance of the BUT. Firstly, the presence of anti-*H. pylori* antibody in the blood may inhibit the production of urease by *H. pylori*. Secondly, serum inhibitors such as enzymes or electrolytes may suppress the urease activity of *H. pylori*. Thirdly, various buffering systems (*e.g.*, albumin, bicarbonate, and phosphate) may interfere with the pH changes of the reagent. In this study, we sought to identify the blood component(s) that interfere with the urease test.

MATERIALS AND METHODS

Materials

Modified Hazell's microtiter urease test. The urease test used in this experiment was modified from that described by Hazell *et al.* (7). Urea (20 g/L), sodium azide (200 mg/L), and sodium phosphate buffer (1 mmol/L) were mixed with bromothymol blue (0.5% weight/volume). The reagent was titrated to pH 5.5 (yellow) before use. A change in color from yellow to blue (pH range, 6.0–7.6), which was measured by the absorbance at 630 nm using a microplate reader (MR5000, Dynatech Laboratories, Chantilly, VA), represented a positive result. Bromothymol blue, instead of phenol red, was chosen as the pH indicator to distinguish the pH color change from blood contamination.

H. pylori. A standard *H. pylori* strain (NCTC 11637) was used in this experiment. The bacteria were cultured in microaerophilic conditions for 3 days and then suspended in normal saline to reach a standardized turbidity (McFarland standard 4). Serial dilutions of the bacterial suspensions confirmed that the concentrations were around 2×10^7

Received Apr. 8, 1998; accepted June 1, 1998.

colony-forming units (CFU)/ml. The bacterial suspensions were freshly prepared each time before use.

Anti-H. pylori *antibody.* Blood samples were collected from two subjects, with and without *H. pylori* infection, respectively, to examine the effects of whole blood and anti-*H. pylori* antibody on the BUT. *H. pylori* status was determined by using the ^{13}C urea breath test and serology (HM-CAP, Enteric Products, New York, NY). The sera were stored at −20°C before use.

Serum inhibitors. Possible inhibitory effects on the BUT by serum electrolytes and enzymes were examined. Ringer's lactated solution, which contains a fluid/electrolyte content similar to human blood, was used in this *in vitro* experiment. In addition, serum that was heated at 60°C for 15 min to denature its enzyme activity, from a subject without *H. pylori* infection, was used to elucidate the effects of serum enzymes on the urease test.

Buffering systems. Several major buffering systems in blood, including serum proteins, hemoglobin, bicarbonate, and phosphates, are responsible for the acid-base homeostasis of the human body. Serum albumin was chosen in this experiment because it is the major protein in blood, is readily available, and has an easily adjusted concentration. The albumin preparation used in this experiment was obtained from pooled human plasma of voluntary blood donors (Hong Kong Red Cross; prepared by CSL Limited, Victoria, Australia).

Experimental procedures

Effect of whole blood on the urease test. One hundred microliters of urease test reagent was added to each of the microtiter wells (Dynatech Laboratories), which contained either human whole blood (30 μl) or distilled water (control). Fifty microliters of *H. pylori* broth culture suspension was then added to each well and incubated at room temperature. Absorbance was measured at 2, 5, 10, 15, 20, 25, 30, 45, 60, and 90 min of incubation. All measurements were triplicated.

Effect of anti-H. pylori *antibody on urease test.* Thirty microliters of: (1) IgG anti-*H. pylori*–positive serum, (2) anti-*H. pylori* antibody-negative serum, and (3) distilled water (control) were added to 100 μl of urease reagent in the microtiter wells. The absorbance was measured serially after the addition of 50 μl of *H. pylori* broth culture suspension, as described. The test was also repeated with the serum diluted 10- and 100-fold.

Effects of serum enzymes and electrolytes-serum inhibitors? The test was repeated by adding 30 μl of: (1) Ringer's lactated solution (McGaw, Ontario, Canada), (2) human anti-*H. pylori*–negative serum, and (3) heat-inactivated anti-*H. pylori*–negative serum to 100 μl of urease reagent and 50 μl of *H. pylori* suspension.

Buffering effects of blood? The effects of serum buffer on the BUT were tested by adding albumin to the ammonia solution and urease test reagents. Bacterial suspension was not used in this part of the experiment to eliminate possible variations in ammonia production by *H. pylori*. Thirty milliliters of human albumin solution at different concentrations (200 g/L, 40 g/L, 20 g/L, and 10 g/L; CSL Limited) were added to 50 μl of the urease test reagents and a fixed quantity of ammonia solution (1% solution, 30 μl). Change in absorbance at different concentrations of serum albumin used was measured.

Statistical analysis

Results were expressed as mean ± SEM. All data were computed using GraphPad Prism software (version 2.00, GraphPad Software, San Diego, CA). One-way analysis of variance with the Tukey's test for *post hoc* comparison was used to establish significant differences in absorbance after the addition of various blood components. Statistical significance was taken at a p value of 0.05 or less.

RESULTS

Effects of whole blood and anti-H. pylori *antibody on the urease test*

The changes in absorbance of the urease test after the addition of whole blood, anti-*H. pylori*–positive and anti-*H. pylori*–negative sera are shown in Figure 1. The changes in absorbance were significantly suppressed by whole blood when compared with control ($p < 0.0001$). Similarly, human sera with or without anti-*H. pylori* antibody produced the same effects on the urease test. However, there was no difference between the anti-*H. pylori*–positive and -negative sera. The inhibitory effects of serum on the urease test were eliminated by dilution to 100-fold (Fig. 2). Thus, the suppression of the urease test was due to certain serum component(s) other than anti-*H. pylori* antibody.

Effects of electrolytes and serum enzymes

The addition of Ringer's lactated solution did not alter the change in absorbance of the urease test when compared with control. However, both heated (denatured enzymes) and unheated sera significantly suppressed the color change of the urease test ($p < 0.0001$; Fig. 3). Thus the suppressive effect on the urease test was neither due to serum electrolytes nor related to an enzymatic component in the serum.

Buffering effect of blood

The changes in absorbance of the urease test in the presence of different concentrations of albumin are shown in Figure 4. With the same concentration of ammonia added to the urease test reagents, the changes in absorbance were inversely related to the concentration of serum albumin used. A marked reduction in colorimetric changes was demonstrated even in the presence of a low concentration of albumin (10 g/L).

DISCUSSION

Eradication of *H. pylori* infection has been shown to prevent recurrent ulcer bleeding, even without using main-

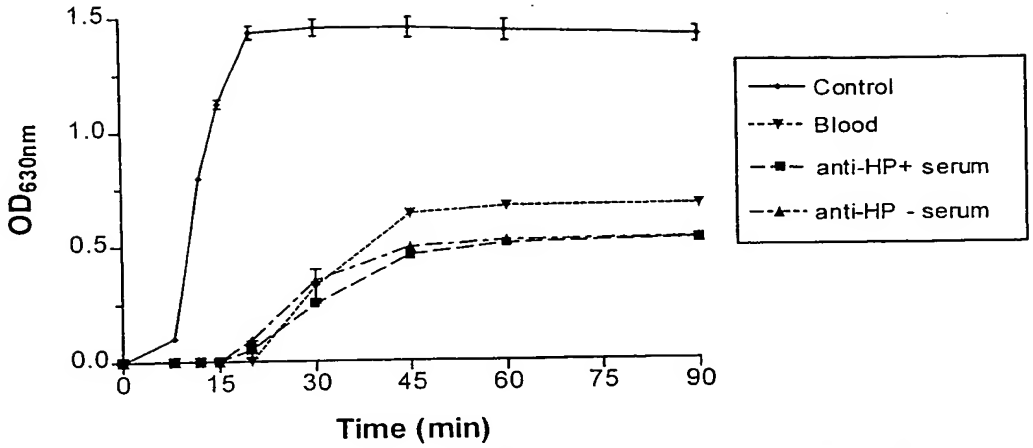

FIG. 1. The changes in color of the urease test after the addition of whole blood, anti-*H. pylori*–positive and -negative sera to *H. pylori* bacterial suspension. $p < 0.0001$ (ANOVA); $p > 0.05$ (blood *vs* anti-*H. pylori*–positive *vs* anti-*H. pylori*–negative serum).

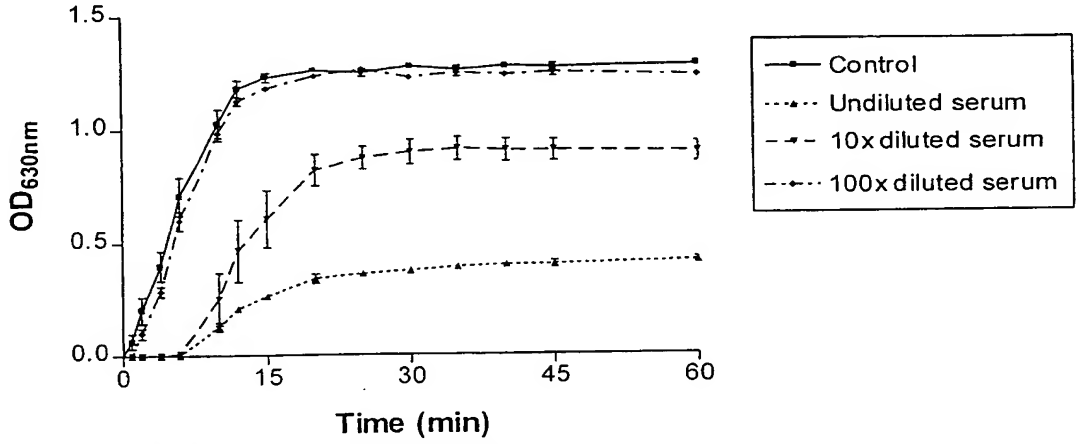

FIG. 2. The color changes of the urease test after the addition of different dilutions of serum to *H. pylori*. $p < 0.0001$ (ANOVA); $p < 0.001$ (control/100× diluted serum *vs* undiluted/10× diluted serum); $p > 0.05$ (control *vs* 100× diluted serum).

tenance acid-suppression therapy (8). Accurate diagnosis of the infection is therefore essential in the management of peptic ulcer bleeding.

Because the results of the biopsy urease test are based on a rise in pH, blood in the stomach and on the biopsy specimen when taken from a case of peptic ulcer bleeding might raise the pH and provide a false-positive result. Perry *et al.* examined the effects of heparinized blood and alcohol on the performance of two BUT, including the CLO test and Hpfast (9). They incubated a serial dilution of urease enzyme with blood or alcohol, and indeed found that heparinized blood enhances the detection of urease. Their results, as the authors concluded, could be interpreted as either improved sensitivity or false-positive results. In fact, the unexpected positive results could be due to the alkalinity of blood on the urease test alone.

On the contrary, several other studies have found reduced sensitivity of the BUT in bleeding peptic ulcers. Colin *et al.* used the serological test as the standard for diagnosis of *H. pylori* infection and studied the accuracy of the BUT (4). The sensitivity of the BUT (31%) was very disappointing. In that study, however, histology and culture also yielded very poor results, raising concerns about the accuracy of their serology test. Lee *et al.* compared the performance of BUT in bleeding *versus* nonbleeding duodenal ulcer (3). In their study, the gold standard for the diagnosis of *H. pylori* infection was based on two of three positive tests on the BUT, histology, and culture. Their results clearly showed a significant drop in sensitivity of the BUT, from 93% in nonbleeding ulcers to 73% in bleeding ulcers, which was consistent with our previous observation (1). The high false-negative rate of the BUT in bleeding duodenal ulcers was further confirmed by Lai *et al.* (2), who used serology as the marker of *H. pylori* infection. Although high-dose omeprazole (80 mg) has been shown to interfere with the accuracy of the BUT (10), this could not explain the low sensitivity

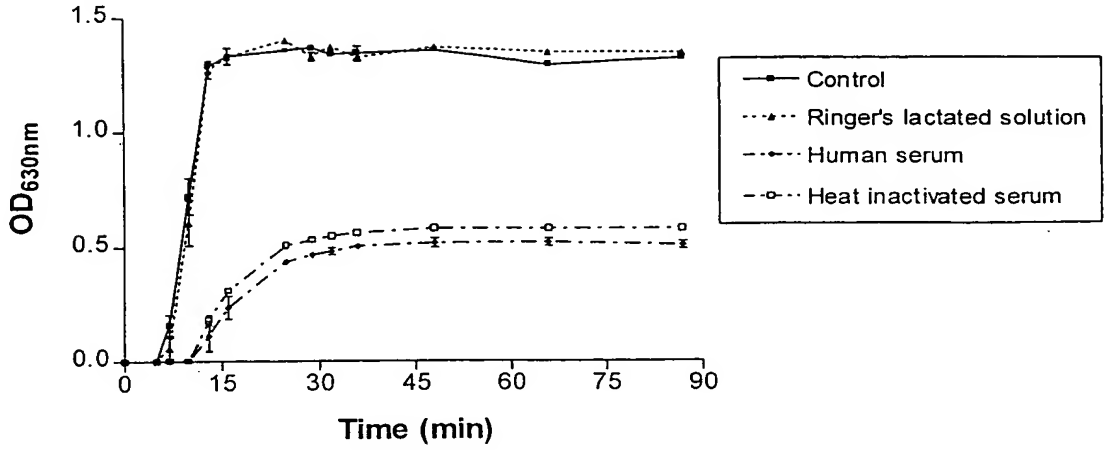

FIG. 3. The changes in color of the urease test after the addition of serum (unheated and heated), and Ringer's lactated solution to *H. pylori*. $p < 0.0001$ (ANOVA); $p < 0.001$ (control/Ringer's lactated solution *vs* heated/unheated serum).

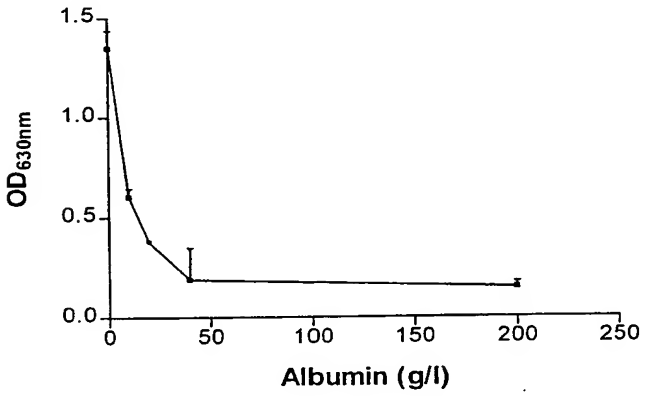

FIG. 4. The changes in color of the urease test after the addition of different concentrations of serum albumin to a fixed amount of ammonia.

of the test as, at least in the latter study (2), patients with recent use of omeprazole and antibiotics were excluded.

Lee *et al.* attempted to study the reasons for the failure of the BUT. They tested the performance of the CLO test, Hpfast, and Pyloritek under various conditions (11). Their results showed that storage temperature, incubation time, and "contamination" with saline, formalin, water, and so on, did not interfere with the results of these commercially available urease tests. On the other hand, after incubation with blood, gastric juice, or bile, the sensitivity of these tests was reduced. The authors attributed this to clotted blood obscuring the color change of the BUT, such as CLO test and Hpfast, but the Pyloritek was relatively unaffected. However, if the reduced sensitivity of the BUT is merely a result of clotted blood obscuring the color change, it would be difficult to comprehend why all three tests failed to detect *H. pylori* even at the high concentration of 10^9 CFU.

In this study, we used cultured *H. pylori* rather than gastric biopsies to ensure that equal amounts of bacteria were present in each microtiter well. Hazell's microtiter urease test was chosen because it is in liquid form, which allows mixing of different test components. The reported sensitivity and specificity were 91% and 100%, respectively (7), which is comparable to the performance of a commercial rapid urease test. Because the urease test is based on colorimetric change, quantitative measurement of light absorbance at specific wavelengths would provide more objective results than visually inspecting the color changes of the commercial urease test. Bromothymol blue, instead of phenol red, was chosen as the pH indicator to avoid the discoloration by blood misinterpreted as positive results.

Our results have confirmed that the presence of blood, or even serum (which is almost colorless) adversely affect the performance of the BUT. The fact that both anti-*H. pylori* antibody-positive and -negative sera produced the same effect on the BUT implies that antibody suppression of urease production by the bacteria is unlikely to be the underlying mechanism. If urease production is not affected, the remaining possibilities are an inhibitor in blood that either suppresses the hydrolytic activity of urease or suppresses the change in pH detected by the indicator. Ringer's lactated solution, which contains a fluid and electrolyte composition similar to that of serum, was found to have no effect on the BUT. Furthermore, serum enzymes could not be an important contributing factor to the false-negative urease test, as the change in absorbance was reduced even when the enzymes were denatured. On the other hand, we showed that the color change of the pH indicator was progressively reduced by an increasing concentration of serum albumin, even in the presence of a constant ammonia level. The only possible explanation left was an inhibitor interfering with the changes in pH, which were detected by the indicator. Though albumin may bind to the pH indicator, the color changes of the pH indicator were a result of the changes in the concentrations of free hydrogen ions, which

are independent of the protein-binding capacity. In addition, similar results were reproduced by using phenol red as the pH indicator (data not shown). Moreover, our observations cannot simply be explained by the background color or the pH of the blood components added to the urease test, as illustrated by the changes in absorbance (630 nm) with time, which was gradual (up to 45 min) rather than instantaneous (Figs. 1–3).

Hence, the buffering effects of blood components, such as bicarbonate, phosphate, hemoglobin, or albumin, which retard the pH changes of the reaction, from an alternative explanation. To study the buffering effects of blood and eliminate the confounding effects of bacterial production or the enzymatic activity of urease, a fixed amount of ammonia was used in the last part of our *in vitro* experiment. We selected albumin to be tested simply because it is the most easily available and adjustable buffer in blood. Our results showed that the suppression of color change on the BUT correlated to the concentration of albumin added. This provides strong evidence that albumin, and possibly other buffer systems in blood, are the main reasons for the failure to detect pH change by the BUT. Albumin, which constitutes the major serum protein, buffers the alkaline effect of ammonia by releasing hydrogen ions. Though only serum albumin was tested in this experiment, there was significant suppression of color change in the urease test by albumin alone. In fact, there was no significant difference observed in the color change of the urease test by the use of serum or serum albumin at a concentration of 40 g/L (data not shown). Therefore, we believe that serum albumin contributes a significant role in interfering with the color changes of the urease test by buffering the pH changes.

Based on these findings, we conclude that in bleeding ulcers, blood reduces the sensitivity of the BUT by interfering with the pH indicator, despite the presence of urease activity. As a result, the BUT, which is a pH-dependent test, should not be used as the only diagnostic test for *H. pylori* infection in patients with bleeding ulcers.

ACKNOWLEDGEMENTS

We are indebted to Dr. Calvin Pang and Dr. Nelson Tang of the Department of Chemical Pathology for their technical advice.

Reprint requests and correspondence: Dr. Joseph J. Y. Sung, Department of Medicine & Therapeutics, Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China.

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APPENDIX

3

ELSEVIER

FEMS Immunology and Medical Microbiology 22 (1998) 283–291

FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY

A lipoteichoic acid fraction of *Enterococcus hirae* activates cultured human monocytic cells via a CD14-independent pathway to promote cytokine production, and the activity is inhibited by serum components

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Received 24 June 1998; received in revised form 24 July 1998; accepted 6 August 1998

Abstract

To elucidate the cellular activation mechanisms of lipoteichoic acid (LTA) compared with those of lipopolysaccharide (LPS), a quantitatively major LTA fraction, QM-1M, was prepared from hot phenol-water extracts of *Enterococcus hirae* (ATCC 9790) by hydrophobic octyl-Sepharose chromatography and by ion-exchange membrane (QMA-Mem Sep 1010) chromatography as a 60% 1-propanol- and 1 M NaCl-eluted fraction. Unlike the reference *Escherichia coli* LPS, QM-1M did not demonstrate any ability to induce cytokines in a human whole blood culture system in this study, whereas QM-1M induced a few cytokines such as interleukin (IL)-8 and tumor necrosis factor-α in human monocytic THP-1 cell and human peripheral mononuclear cell (PBMC) cultures in the absence of serum. Fetal calf and human sera decreased the above cytokine induction by QM-1M in THP-1 and PBMC cultures, whereas sera increased activities of the reference LPS. IL-8 induction in the absence of serum in response to QM-1M was demonstrated to proceed through a CD14-independent pathway unlike the reference LPS.

Keywords: Lipoteichoic acid; *Enterococcus hirae*; Interleukin-8; Tumor necrosis factor-α; CD14; Monocyte

1. Introduction

Lipoteichoic acids (LTAs) are cell surface amphiphiles widely distributed in Gram-positive bacteria [1]. Several studies of the immunobiological activities of LTA have indicated that LTA shares various bioactivities with endotoxic lipopolysaccharide (LPS) from Gram-negative bacteria (reviewed in [1–3]). More recent evidence has suggested that LTA and LPS share CD14 as a common receptor system. Cleveland et al. [4] demonstrated that LTA preparations from *Staphylococcus aureus* and *Streptococcus pyogenes* induced interleukin (IL)-12 through the

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0928-8244/98/$19.00
PII: S0928-8244(98)00097-2

CD14-dependent pathway in human monocytic THP-1 cell cultures. Hattor et al. [5] also reported that the induction of nitric oxide (NO) synthesis and an isoform of NO synthase gene expression by a mouse macrophage cell line (J774) in response to LTA was significantly inhibited by an anti-mouse CD14 monoclonal antibody (MAb). In addition, Renzi and Lee [6] reported that LTA from various bacterial species stimulated ICAM-1 expression by human umbilical vein endothelial cells in a serum-dependent manner, as does LPS, and that an anti-CD14 MAb inhibited the ICAM-1 expression by the cells in response to LTA as well as LPS.

In a previous study [7], we prepared two LTA fractions, LTA-1 and -2, from *Enterococcus hirae* ATCC 9790 according to the method of Fischer et al. [8]. The quantitatively minor fraction LTA-2 was bioactive, mainly in murine in vitro and in vivo assay systems, showing the ability to induce a variety of cytokines and an antitumor effect. In contrast, the quantitatively major fraction LTA-1 was scarcely active in the same assays. We also found that LTA-related compounds which were chemically synthesized by mimicking the fundamental structures of LTA-1 and -2 [9,10] were bio-inactive as far as tested, except for antigenicity [7]. The bioactive LTA-2 fraction was not satisfactorily homogeneous, and thus Suda et al. [11] tried to isolate the bioactive component of LTA-2 in terms of the IL-6- and tumor necrosis factor (TNF)-α-inducing activities of LTA-2 observed in a human whole blood culture system. Although they obtained five biologically active, high molecular mass glycolipids at a total yield of 6% of LTA-2, the chemical analytical results led them to suggest that the component responsible for the observed bioactivities might be a novel compound different from those of conventional LTAs in chemical characteristics [11]. A later study by Hashimoto et al. [12] further showed that a major LTA fraction prepared from *E. hirae* ATCC 9790, which shared the chemical structure with that reported by Fischer [1], was devoid of cytokine-inducing activities in a human whole blood culture system. A view similar to that described by Suda et al. was advanced by Kusunoki et al. [13], who attempted to isolate a bioactive component from a commercial LTA preparation derived from *S. aureus*, since a purified LTA fraction separated by hydrophobic chromatography lacked the capacity to induce cytokines in human whole blood, and the bioactive compound was recovered in a fraction other than the above LTA fraction.

These studies suggest that LTA structures, in a narrow sense (such as those described by Fischer [1]), might be incapable of exhibiting various bioactivities in human whole blood culture systems via a CD14- and serum-dependent pathway. Nevertheless, the above findings do not necessarily exclude the possibility that the quantitatively major *E. hirae* LTA fraction exhibits various bioactivities via a serum- and CD14-independent pathway. The objective of this study was to examine the possible activation of human monocytic cells via a serum- and CD14-independent pathway by a quantitatively major LTA preparation from *E. hirae*, which was reported to be inactive in the human whole blood assay system used by Suda et al. [11] and Hashimoto et al. [12].

2. Materials and methods

2.1. Major E. hirae LTA fraction, QM-1M

A test specimen of a quantitatively major LTA fraction, QM-1M, from *E. hirae* ATCC 9790 prepared principally according to the method of Hashimoto et al. [12] was supplied by Y. Suda and S. Kusumoto (Graduate School of Science, Osaka University, Osaka, Japan). Briefly, delipidated cells of *E. hirae* were extracted with hot phenol-water. The extract was digested with DNase/RNase to give a crude LTA fraction. The crude fraction was fractionated with Octyl-Sepharose (Pharmacia, Uppsala, Sweden) by serial elution with 0.1 M acetate buffer (pH 4.5) containing 15 and 60% 1-propanol to give fractions BOS15 and 60, respectively (the yield of both was about 30% of the crude LTA). A specimen of BOS60 dissolved in 0.01 N acetate buffer containing 35% 1-propanol was applied to an ion-exchange membrane, OMA-Mem Sep 1010 (PerSeptive Biosystems, Framingham, MA, USA). Unbound (pass-through) material was recovered by washing out the membrane with the above buffer to give a fraction capable of inducing IL-6 in human whole blood cultures. The materials bound to the membrane were

Table 1
Chemical compositions of QM-1M and QM-I[a]

	QM-1M		QM-I[b]	
	Wt%	μmol mg^{-1}	Wt%	μmol mg^{-1}
Phosphate	11.0	1.7	11.5	1.8
Glycerol	18.6	2.1	18.7	2.1
Glucose	58.1	3.6	42.4	2.6
Fatty acids	7.3		7.6	
16:0		0.10		0.10
16:1		0.01		0.02
18:0		0.01		0.03
18:1		0.17		0.13

[a]Analytical methods were described previously [11].
[b]Quoted from Hashimoto et al. [12].

eluted with the buffer containing 35% 1-propanol and 1 M NaCl to give a quantitatively major LTA fraction, QM-1M, which was incapable of inducing IL-6 in human peripheral blood cultures. The recovery rate of the QM-1M fraction was 44% of BOS60. The chemical composition of the QM-1M preparation which served as a test stimulant in the present study was essentially the same as that of QM-I reported by Hashimoto et al. [12] (Table 1).

2.2. Other reagents and cells

An LPS specimen prepared from *Escherichia coli* O111:B4 by the hot phenol-water extraction method was purchased from Difco Laboratories (Detroit, MI, USA) and used as a reference stimulant. The anti-CD14 MAb MY-4 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The human monocytic cell line THP-1 was obtained from the Health Science Research Resources Bank (Tokyo, Japan).

2.3. Cytokine induction in cell cultures

THP-1 cells (2×10^6) were seeded in a 24-well culture plate and cultured in duplicate in 1 ml of RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) with or without 1% fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY, USA) and in the presence or absence of test stimulants in a CO_2 incubator. After 24 h, culture supernatants were collected and stored at −80°C until use. Cytokine induction in human peripheral whole blood and peripheral blood mononuclear cell (PBMC) culture systems was performed as described previously [14]. Briefly, in the whole blood cultures heparinized human peripheral whole blood (25 μl) obtained from a healthy adult volunteer was cultured in RPMI 1640 medium (75 μl) with or without test materials in a 96-well culture plate (Corning Laboratory Sciences, Corning, NY, USA) in triplicate. In the case of PBMC cultures, human PBMC prepared from heparinized peripheral blood by Ficoll-Isopaque centrifugation were cultured at a density of 4×10^5 cells per 200 μl of RPMI 1640 medium in a 96-well culture plate for 24 h with or without test materials and in the presence or absence of FCS or autologous human serum. After the cultivation, the triplicate culture supernatants were pooled and stored. Various cytokines in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) kits (Otsuka Pharmaceutical Co., Tokushima, Japan; and Biosource International, Camarillo, CA, USA). THP-1 cell cultures were examined for the following cytokines: IL-1α; IL-1β; IL-2; IL-6; IL-8; TNF-α; macrophage colony-stimulating factor (M-CSF); granulocyte colony-stimulating factor (G-CSF); granulocyte-macrophage colony-stimulating factor

Table 2
Inability of QM-1M to induce cytokines in human whole blood cultures[a]

Test material		IL-6 (U ml^{-1})	IL-8 (pg ml^{-1})	TNF-α (pg ml^{-1})
Medium alone		ND[b]	66 ± 10	ND
LPS[c]	(0.01 μg ml^{-1})	11 488 ± 1785	1322 ± 130	925 ± 106
QM-1M	(100 μg ml^{-1})	ND	99 ± 18	ND
	(10 μg ml^{-1})	ND	55 ± 21	ND

[a]The results are representative of three different experiments.
[b]Not detected.
[c]From *E. coli* O111:B4.

(GM-CSF); interferon (IFN)-α; and IFN-γ. For technical reasons, only selected cytokines were measured in the human peripheral whole blood and PBMC cultures.

2.4. CD14 expression by THP-1 cells

The expression of membrane CD14 (mCD14) on THP-1 cells was analyzed by flow cytometry (Nippon Becton Dickinson Co., Tokyo). THP-1 cells (2×10^6) were seeded in a 24-well culture plate, and cultured in 1 ml of RPMI 1640 medium without FCS in the presence of 10 μg ml^{-1} of QM-1M or the reference LPS. After a 36-h cultivation, cells were incubated with the MAb MY-4 at 4°C for 1 h, washed three times with phosphate-buffered saline (PBS), pH 7.4, stained with FITC-conjugated anti-mouse immunoglobulin, and then analyzed by flow cytometry.

2.5. Miscellaneous

All test specimens for cytokine assays were stored at −80°C until tested. Most of the assays were carried out in duplicate or triplicate. The range of the cytokine levels induced in triplicate assays was within 20% of the respective mean value. In some experiments, the statistical significance of the differences between each test and the respective control was determined by Student's *t*-test. Most experiments were carried out more than twice, and representative results are presented.

3. Result

3.1. Inability of QM-1M to induce cytokines in human whole blood cultures

As shown in Table 2, QM-1M was scarcely capable of inducing TNF-α and IL-8 as well as IL-6 in human whole blood cultures even at a high concentration (100 μg ml^{-1}), under the experimental conditions in which the reference LPS markedly induced these cytokines at a low concentration (0.01 μg ml^{-1}). These data strongly suggest that the test QM-1M preparation was not significantly contaminated with exogenous endotoxins or other bioactive materials such as those described by Suda et al. [11] and Kusunoki et al. [13].

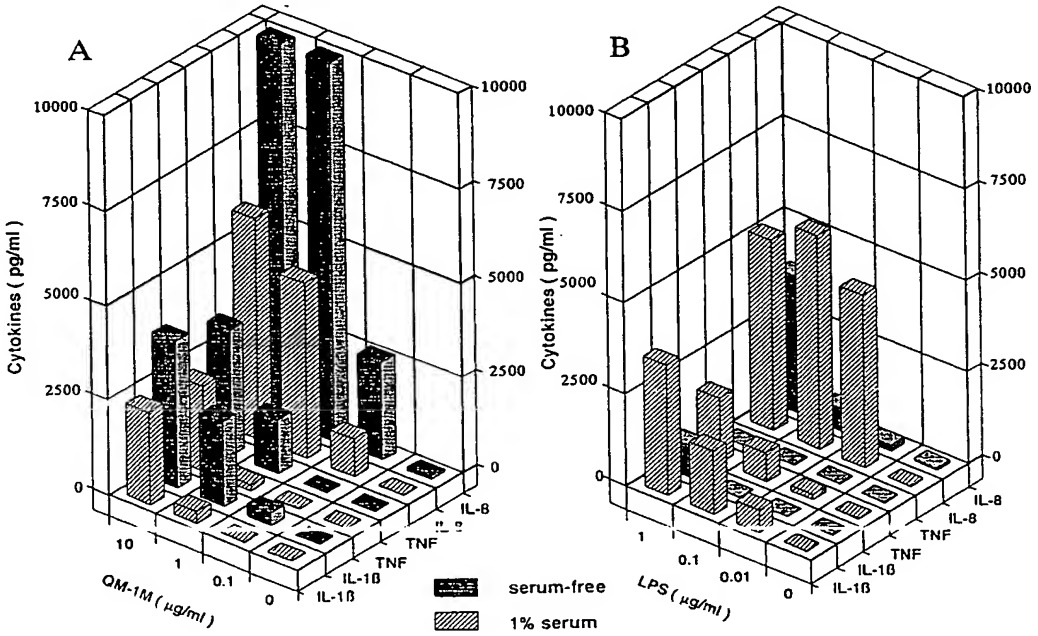

Fig. 1. IL-1β-, IL-8-, and TNF-α-inducing activity of QM-1M (A) and LPS (B) in THP-1 cell cultures (2×10^6 ml^{-1}) in the presence of FCS (representative of five different experiments).

3.2. QM-1M induces various cytokines in THP-1 cell cultures, and serum components inhibit the cytokine induction by QM-1M

We examined the cytokine-inducing abilities of QM-1M in a human monocytic cell line, THP-1 cultures. In the absence of FCS, QM-1M was capable of inducing IL-1β, TNF-α and IL-8; the IL-8 induction was the most marked (Fig. 1A). The dose-response curve of IL-8 induction by QM-1M is shown in Fig. 2. QM-1M induced IL-8 at 0.1 μg ml^{-1}, and the activity increased dose-dependently to 10 μg ml^{-1}. An overdose depression was noted at 100 μg ml^{-1} due to unidentified causes. QM-1M also induced IL-1α, IL-6, G-CSF and M-CSF, but the extent of induction was less than those of the other cytokines and showed fluctuations from one assay to another. IL-2, GM-CSF, IFN-α and IFN-γ were scarcely induced by QM-1M in this system (data not shown). The comparison of the data of QM-1M and LPS in Fig. 1 illustrates that in the absence of serum, QM-1M generally exhibited stronger cytokine-inducing activities than the reference LPS at comparable test dose levels (1.0 and 0.1 μg ml^{-1}). In the presence of 1% FCS, the cytokine-inducing activities of LPS were markedly increased, presumably because of the existence of cofactors such as LPS-binding protein (LBP) in the serum (Fig. 1B). The cytokine-inducing activities of QM-1M decreased (Fig. 1A). Similar inhibitory effects of FCS on the cytokine-inducing activities of QM-1M were observed in an assay using the murine macrophage cell line J774.1 (data not shown). The presence of human serum in the assay system likewise decreased the cytokine-inducing activities of QM-1M in THP-1 cultures (data not shown). The findings described above strongly suggest that QM-1M activates human and murine monocytic cells in a manner different from LPS.

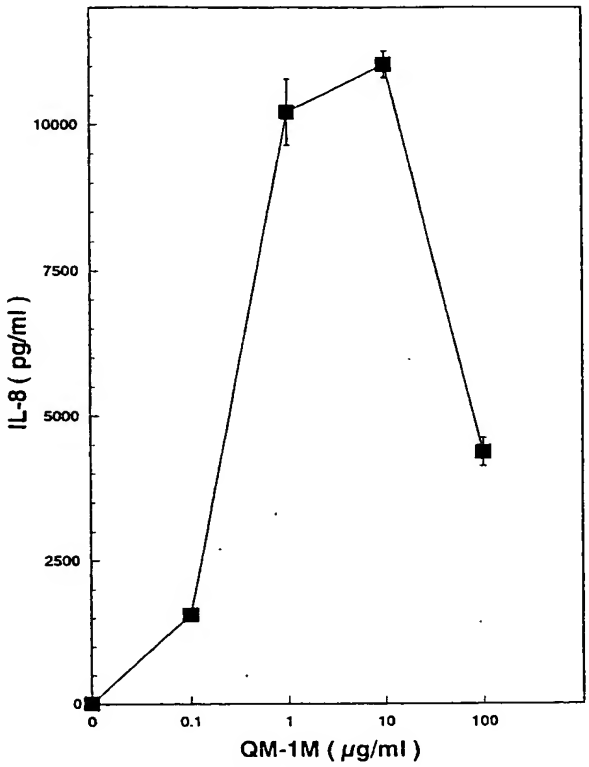

Fig. 2. The dose-response curve of the IL-8 in response to QM-1M in THP-1 (2×10^6) cell cultures in the absence of serum (representative of five different experiments).

Table 3
QM-1M induces IL-8 and TNF-α in human PBMC cultures in the absence of serum, but not in the presence of serum[a]

Test material	Concn (μg ml^{-1})	Serum-free	1% AS[b]	10% AS	1% FCS	10% FCS
IL-8 response (ng ml^{-1})						
Medium alone		57 ± 2.2	107 ± 11.8	165 ± 53	85 ± 9	63 ± 6.1
QM-1M	1	120 ± 8.3**	81 ± 18.0	164 ± 1.9	109 ± 3.5	41 ± 0.5
LPS[c]	0.1	98 ± 5.8*	220 ± 10.9*	209 ± 6.1	212 ± 14.7**	144 ± 2.2**
TNF-α response (pg ml^{-1})						
Medium alone		86.3 ± 5.1	34.8 ± 3.9	3.9 ± 0.9	NT[d]	23.7 ± 4.1
QM-1M	1	149.5 ± 0.5**	27.5 ± 1.0	3.9 ± 0.4	NT	22.7 ± 1.2
LPS	0.1	577 ± 139*	185 ± 28*	16.1 ± 1.9*	NT	118 ± 0.6**

[a]Representative of three different experiments. Significance with Student's *t*-test: $*P \leq 0.05$, $**P \leq 0.01$.
[b]Autologous serum.
[c]From *E. coli* O55:B5.
[d]Not tested.

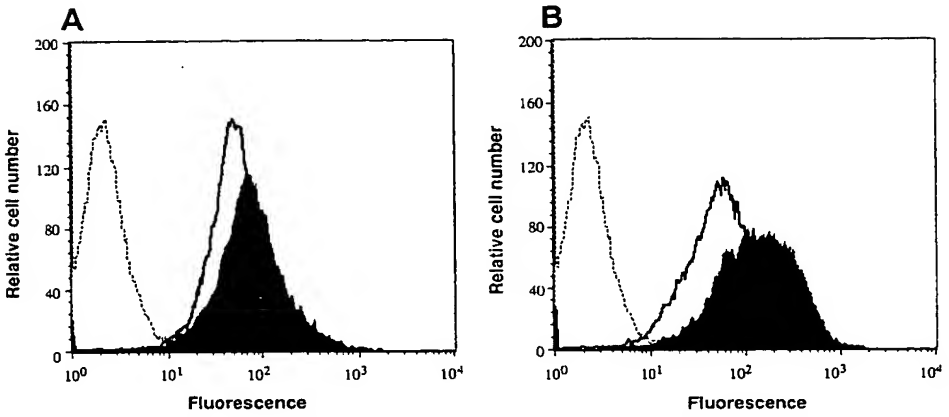

Fig. 3. Effects of QM-1M (A) and LPS (B) on mCD14 expression by THP-1 cells. Cells stained with second antibody only (dotted line) or the anti-CD14 MAb MY-4 plus the second antibody (solid line). Open area bounded by hatched line, cells cultured in medium alone; closed area, cells cultured with a stimulant. The results are representative of two different experiments.

3.3. *QM-1M induced IL-8 and TNF-α in human PBMC cultures in the absence of serum, but not in the presence of serum*

We then examined the possible cytokine-inducing ability of QM-1M in human PBMC cultures. Table 3 shows that QM-1M was capable of inducing IL-8 and TNF-α by the stimulation of human PBMC cultured without serum. The extent of stimulation in terms of the net increase in the levels of either IL-8 or TNF-α was small, but the increase was significant. The reliability of data was further supported by the finding that the addition of either autologous human serum or FCS to PBMC cultures abolished the cytokine-inducing activity of QM-1M, but this was not the case with the reference LPS.

3.4. *QM-1M activates THP-1 cells through a CD14-independent manner*

Cleveland et al. [4] reported that LTA preparations from *S. aureus* and *S. pyogenes* induced IL-12 in THP-1 cultures through a CD14-dependent pathway. Thus, we examined the possible involvement of mCD14 in the activation of THP-1 cells by QM-1M. We first analyzed the CD14 expression on THP-1 cells by flow cytometry using the anti-CD14 MAb MY-4. Fig. 3 shows that although the THP-1 cells spontaneously expressed mCD14 to a considerable extent, LPS treatment for 36 h powerfully upregulated the expression of mCD14 by the cells. In contrast to this control experiment, QM-1M only slightly upregulated the mCD14 expression by the cells. We then examined the effects of the anti-CD14 MAb on IL-8 induction by QM-1M and LPS (Table 4). Under the experimental conditions where the MAb MY-4 definitely inhibited IL-8 induction by LPS in the presence of 1% FCS (such inhibitory effects were marginal in serum-free conditions), no significant inhibiting effect of the MAb on

Table 4
Effects of the anti-CD14 monoclonal antibody MY-4 on the IL-8-inducing activity of QM-1M and LPS in THP-1 cells[a]

Test material	Concn (μg/ml)	IL-8 (pg/ml)		
		Serum-free	1% FCS	
Medium alone		31 ± 11	22 ± 3	
QM-1M	1	1,707 ± 32**	118 ± 10**,++	
LPS	1	304 ± 73*	1,834 ± 103**,++	
MY-4	10	74 ± 18	44 ± 2*	ns
QM-1M + MY-4	1 + 10	2,012 ± 163**	73 ± 11*,++	s
LPS + MY-4	1 + 10	228 ± 18**	438 ± 65*,+	
		ns	ns	

[a]Representative of three different experiments. Significant differences from the respective medium alone control (*,**) and from the respective serum-free culture (+,++) by Student's *t*-test (*,+ $P \leq 0.05$, **,++ $P \leq 0.01$). The differences were statistically significant (s) and not significant (ns) by Student's *t*-test ($P \leq 0.05$).

IL-8 induction by QM-1M was seen in the presence or absence of FCS.

4. Discussion

In this study, we demonstrated that a quantitatively major LTA fraction, QM-1M, was capable of exhibiting various cytokine-inducing activities in a human monocytic cell line, THP-1, in the absence of serum under experimental conditions in which the possibility that observed bioactivities are due to contamination with extraneous endotoxins can be excluded (Fig. 1). Considerable evidence indicates that LPS activates various cells mainly through CD14-dependent mechanisms (reviewed in [15]). Experimental evidence (Table 4) indicated that QM-1M stimulated THP-1 cells to produce IL-8 through a CD14-independent pathway (unlike the reference LPS), although QM-1M slightly upregulated mCD14 on THP-1 cells (Fig. 3) as suggested by Landmann et al. [16]. In contrast, there are reports of CD14-dependent activities of LTA preparations [4–6]. We also found that high mCD14-expressing human gingival fibroblasts released IL-8 in response to QM-1M and *Bacillus subtilis* LTA, whereas low mCD14-expressing human gingival fibroblasts did not (Sugawara, S., Arakaki, R. and Takada, H., unpublished). These findings indicate that LTA specimens exhibited activities through both CD14-dependent and -independent pathways. Whether an active structural entity responsible for both activities is the same or not is unclear at present. A possible interaction of the test LTA preparations with mCD14 was suggested by the observation of Kusunoki et al. [13] that a purified LTA obtained from *S. aureus* bound soluble CD14 (sCD14) and antagonized the IL-6-inducing activity of LPS in human astrocytoma U373 cultures, probably by competition with LPS binding for sCD14. We also observed the antagonistic effect of some LTA preparations against LPS in a human gingival fibroblast culture system (Sugawara, S., Arakaki, R. and Takada, H., unpublished). These observations suggest that LTA is capable of binding with CD14, but the binding may result in signal induction in some cases and may result in antagonistic effects (to agonists such as LPS) in other cases.

We also found that a marked cytokine induction by QM-1M in THP-1 cells and human PBMC was reduced by the presence of FCS or human serum, unlike LPS (Fig. 1 and Table 3). Standiford et al. [17] also reported that definite IL-8-inducing activity of LTA from *S. aureus* and *S. pyogenes* from human PBMC probably in the absence of serum, although they did not examine the influence of serum. The inability of QM-1M to induce cytokines in a human whole blood culture system might be attributable to the high concentration (more than 10%) of human serum in the assay system. One of the possible inhibitory factors is the anti-LTA antibodies which are known to be common in healthy human serum (reviewed in [1]). Serum specimens of the donors of whole blood cultures in the present and previous studies contained considerable levels of anti-LTA antibodies (Arakaki, R., Sato, M. and Takada, H., unpublished) which might influence the bioactivities of LTA preparations. Mancuso et al. [18] reported that anti-LTA antibodies enhanced the release of cytokines by human monocytes sensitized with LTA. It is well known that serum factors such as LBP [19–21], septin [22] and sCD14 [23,24] are required for efficient cellular activation. These proteins and other LPS-recognizing agents such as bactericidal permeability-increasing protein [25] and high-density lipoprotein [26,27] might also interact with LTA and interfere with the exhibition of its bioactivities. In this context, Greenberg et al. [28] reported that bovine macrophage scavenger receptor which binds LPS also recognized specified LTA structures. The following serum factors have been demonstrated to bind with LTA as well as LPS: a 28-kDa protein in murine sera [29]; albumin [30]; and sCD14 [13]. A study examining the possibility that sCD14 is involved in the inhibitory activity of serum against the cytokine-inducing activities of QM-1M is in progress at Tohoku University, using recombinant sCD14.

Our previous [12] and present chemical analyses (Table 1) suggest that the QM-1M fraction is predominantly composed of molecules whose structure in principle corresponds to that proposed for LTA-1 from *Streptococcus faecalis* (*E. hirae*) ATCC 9790 by Fisher et al. [1,8]. The bioactivities exhibited by the QM-1M fraction in this study may be attributable to a main compound with an LTA-1 structure; how-

ever, since the QM-1M fraction was not homogeneous, the possibility should be considered that the bioactivities of the QM-1M fraction are attributable to an amphiphile different in chemical structure from LTA-1 molecules. In this context, we note that a purified *Streptococcus sanguis* LTA, the structure of which was reported to be comparable to that of LTA-1 [31], was scarcely active in the same assay systems adopted here (Arakaki, R. and Takada, H., unpublished). Further studies are in progress at Osaka University on bioactive structure(s) in LTA preparations from *E. hirae* ATCC 9790. We reserve a final conclusion on the chemical entity responsible for the bioactivities exhibited by LTA preparations including the QM-1M fraction until it is completely chemically defined, preferably chemically synthesized LTA molecules.

Acknowledgments

Grateful acknowledgment is made to Professor S. Kusumoto and Dr. Y. Suda (Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka, Japan) for kindly supplying the QM-1M fraction and its chemical analytical data. We thank Drs. K. Matsushita (Kagoshima University Dental School) and M. Takahashi (Tohoku University School of Dentistry) for their help with the ELISA assay. We also thank D. Mrozek (Medical English Service, Kyoto, Japan) for reviewing this paper and Drs. T. Yoshida and T. Matsuno (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) for their continuing interest and encouragement. This work was supported in part by grants-in-aid (07671977 and 10470378) for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan and by a grant-in-aid for scientific research from Chugai Pharmaceutical Co., Ltd.

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